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Sensitivity in Horseradish Peroxidase Neurohistochemistry: A Comparative and Quantitative Study of Nine Methods[1]

MAREK-MARSEL MESULAM AND DOUGLAS L. ROSENE

Bullard and Denny-Brown Laboratories of the Harvard Neurological Unit (M-M.M., D.L.R.), Beth Israel Hospital, 330 Brookline Avenue, Boston, Massachusetts 02215, and the Boston University School of Medicine, Department of Anatomy (D.L.R.), Boston, Massachusetts

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Nine currently available methods for HRP neurohistochemistry have been compared with each other on matching tissue sections from four rats and four rhesus monkeys. The nine methods investigated in this report are the diaminobenzidine (DAB) procedures of LaVail JH and LaVail MM (J Comp Neurol 157:303, 1974), of Adams JC (Neuroscience 2:141, 1977) and of Streit P and Reubi JC (Brain Res 126:530, 1977); the benzidine dihydrochloride (BDHC) procedures of Mesulam M-M (J Histochem Cytochem 24: 1273, 1976) and of De Olmos J and Heimer L (Neurosci Lett 6:107, 1977); the *o*-dianisidine (O-D) procedure of De Olmos J (Exp Brain Res 29:541, 1977); the *p*-phenylenediamine dihydrochloride and pyrocatechol (PPD-PC) procedure of Hanker JS *et al.*, (Histochem J 9:789, 1977) and the tetramethyl benzidine (TMB) procedures of Mesulam M-M (J Histochem Cytochem 26:106, 1978) and of De Olmos J *et al.* (J Comp Neurol 181:213, 1978). Quantitative comparisons were based on counts of retrogradely labeled perikarya. The extent of anterograde transport and the size of the injection site were also compared at a more qualitative level. The results indicate that one TMB procedure (Mesulam M-M, J Histochem Cytochem 26:106, 1978) is distinctly superior to each of the other eight procedures in the number of labeled perikarya that it can demonstrate. Furthermore, these differences are statistically significant at better than the 0.05 level of confidence. Differences in sensitivity are most evident when the perikarya contain small quantities of transported HRP. The same TMB method also demonstrates more anterograde transport and a larger injection site than all the other procedures. If less sensitive procedures are employed, afferent or efferent connections that are clearly demonstrated by this TMB procedure are either underestimated or completely overlooked. It is suggested that sensitivity in HRP neurohistochemistry is determined by multiple factors which include the method of fixation, post-fixation storage, the choice of chromogen, the incubation parameters, the type of HRP enzyme that is administered, and the postreaction treatment.

In horseradish peroxidase (HRP) neurohistochemistry, the threshold for visualizing transported HRP is dependent on a host of variables including the method of fixation, the nature of the chromogen and the choice of incubation parameters. These variables control the deposition of reaction-product at sites of HRP activity and are of significant practical value to the neuroanatomist. Indeed, neural connections which are readily visualized when one set of histochemical parameters is employed, may totally elude detection in experiments that use excessive fixation, weak chromogens or suboptimal incubation parameters (De Olmos and Heimer, 1977; Mesulam, 1976, 1978; Mesulam and Rosene, 1977; Rosene and Mesulam, 1978).

Initial experiments with HRP neurohistochemistry almost exclusively employed the LaVail and LaVail (1974) modification of the Graham and Karnovsky (1966) method. Recently, several new procedures have been described that claim a level of sensitivity or specificity for detecting transported HRP superior to the LaVail and LaVail m thod (Adams, 1977; De Olmos, 1977; De Olmos and Heimer, 1977; De Olmos *et al.*, 1978; Hardy and Heimer, 1977; Hanker *et al.*, 1977; Mesulam, 1976, 1978; Streit and Reubi, 1977). While some of these newer procedures have been rigorously compared to the LaVail and LaVail method, they have not been compared among themselves in any systematic fashion. Hence, the individual investigator who wants to optimize experiments with HRP is now faced with a difficult choice. In order to reduce this difficulty, this article reports the outcome of systematic and quantitative comparisons among nine methods currently available for HRP neurohistochemical experiments. These comparisons show that the sensitivity of one method which employs 3,3′, 5,5′ tetramethyl benzidine as the chromogen (Mesulam, 1978) is superior to the other eight.

METHODS

Observations from four rhesus monkeys (M1–M4) and four rats (R1–R4) are reported. In different experiments injections of 0.05–0.2 μl of a 20% aqueous solution of HRP (Sigma VI, Sigma Chemical Co., St. Louis, MO. or Miles Labs, Inc., Kankakee, IL.) were made in distinct sites such as the hippocampus (R1), the entorhinal cortex (R4), the olfactory bulb (R2), neocortex (R3, M2, M3, M4) or the corpus callosum (M1). After a survival period of 48 hr, the animal

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was perfused according to fixation Procedure II of Rosene and Mesulam (1978). After the perfusion, the brain was removed, placed in a solution of a 10% sucrose in 0.1 M phosphate buffer at pH 7.4 and 4°C for 2 to 12 hr and then cut into 40 μ sections on a freezing microtome. The sections were collected in the phosphate buffer without sucrose.

Nine distinct histochemical methods were employed and these are listed in Table 1. In the following text, the different methods will be designated by the abbreviations in the left hand column of Table 1. These abbreviations are based on the chromogen employed by the individual method. Where more than one method employs the same chromogen, the initials of the authors have been added. As published, many of these procedures suggest a range for some of the incubation parameters. Table 1 specifies the exact values within these ranges which were employed in the present investigation. In all cases, the choice was made to maximize sensitivity.

Four rats (R1–R4) were used for the systematic and quantitative experiments. In these rats, the brains were serially sectioned into 9 adjacent series of sections. One series from each of the four cases was processed according to one of the 9 procedures listed in Table 1. All 4 series that were processed according to the same procedure were simultaneously reacted in separate compartments of the same reaction vessel in order to eliminate surreptitious between-subject differences in handling. All sections were processed on the day after they were sectioned and were counterstained 1 day later. Procedures which yield a blue or green reaction product were counterstained with neutral red while those which yield a brown reaction product were counterstained with thionin.

In these four rats, 12 anatomical structures containing retrogradely transported HRP were selected for quantitative analysis of labeled neurons. The extent of anterograde transport and the size of the HRP injection were also qualitatively compared. For each of the 12 sites, 3–6 consecutive sections spanning the relevant cerebral structures were used for counting labeled perikarya. Thus, each section used for counting HRP-labeled neurons had a closely matching counterpart in each of the 9 procedures. Therefore, anatomical variations in the density of labeling could not have influenced the results since each of the 9 series contained a representative sampling of the pertinent region. These experiments enabled a systematic comparison of all 9 procedures in matching sections from the same animal so that variations in injection size, injection locus, survival time, method of fixation or other intervening details in handling could not have affected the validity of the results.

Each of two experimenters counted labeled perikarya independently with the help of a ×10 ocular fitted with a scored reticle, a ×20 objective and a 100 W quartz-halogen light source. Bright-field and dark-field illumination were used as needed in order to maximize the detection of labeled perikarya. In 3 of the 12 counting sites, only the TMB(m) procedure contained a significant number of labeled perikarya and these structures were excluded from the statistical analysis. In the other 9 structures, the median number of labeled perikarya from the two independent counts was determined and the 9 procedures were then ranked from 1 to 9 for each structure. A summary rank order was determined across all 9 procedures by computing the average rank. A Friedman nonparametric analysis of variance for rank sums (Bradley, 1968) was then applied to determine if differences in the final summary ranks were statistically significant. In this case and all others, nonparametric statistical procedures were employed since cell counts constitute an interval measure and do not meet the assumptions of standard parametric statistical analysis. Furthermore, the statistical significance of differences between individual procedures was calculated using the method for multiple comparisons described by McDonald and Thompson (1967). Because such posthoc multiple comparisons could not statistically resolve all the between-procedure differences, in further independent experiments, pair-wise comparisons were conducted between the TMB(m) procedure and the DAB(sr), BDHC(dh), BDHC(m) and TMB(dhh) procedures (Cases M1–M4). The statistical significance of these comparisons was determined by the Wilcoxon matched-pairs, signed rank sum test (Bradley, 1978). Finally, interobserver reliability was estimated by Spearman's rank-order correlation (Siegel, 1956) and ranged from 0.96 to 1.0 (median = 0.99, all correlations $p < 0.01$); and intraobserver reliability was found to range from 0.97 to 1.0 (median = 0.99, all correlations $p < 0.01$) based upon sections which were counted twice.

RESULTS

Retrograde Transport: In four rats (R1–R4), all 9 procedures listed in Table 1 were performed on 9 matching series of sections from each case. In 9 of the 12 sites analyzed, each of the 9 procedures demonstrated perikaryal labeling. However, striking differences in the numbers of labeled neurons could be demonstrated (Fig. 1).

The 9 procedures were ranked on the basis of labeled perikarya at each of the 9 counting sites (Table 2). An overall summary rank was computed and shown to be significantly different from a chance distribution at the 0.005 level of confidence by the Friedman nonparametric analysis of variance (Bradley, 1968). The summary ranking of sensitivity placed the TMB(m) procedure 1st; the DAB(sr) procedure 2nd; the BDHC(dh) procedure 3rd; the BDHC(m) procedure 4th; the TMB(dhh) procedure 5th; the DAB(ll) procedure 6th; the O-D procedure 7th; the DAB(a) procedure 8th; and the PPD-PC procedure 9th. As shown by the average ranks presented in Table 2, some of the procedures were closely ranked while others clearly differed. Using the method of McDonald and Thompson, pair-wise multiple comparisons were conducted to maintain an experiment-wise error rate of 0.05. Thus the TMB(m) procedure was significantly superior to the PPD-PC, DAB(a), O-D, and DAB(ll) methods, but did not differ significantly from the DAB(sr) method and approached significance in comparison with BDHC(dh), BDHC(m) and TMB(dhh). Furthermore, similar comparisons showed that the DAB(sr) method was also significantly superior to the three lowest ranked procedures, PPD-PC, DAB(a) and O-D, and marginally superior to the DAB(ll) method, but did not differ significantly from the others. Finally, the only other significant pair-wise difference indicated that the BDHC(dh) method was superior to the PPD-PC procedure. Because the posthoc multiple comparison procedure is extremely and appropriately conservative, it could not resolve differences between procedures occupying adjacent summary rankings. Therefore, in order to more thoroughly test the apparent superiority of the TMB(m) procedure it was compared separately in 4 additional experiments to the other 4 relevant procedures, DAB(sr), BDHC(dh), BDHC(m) and TMB(dhh) (Table 3). These results were compared using the Wilcoxon matched-pairs, signed rank-sum procedure (Bradley, 1968) and indicated that the TMB(m) procedure is significantly superior at the 0.03 level to each of the other 4 procedures in Table 3.

In certain counting sites such as the subiculum in case R4, the differences between the TMB(m) procedure and the others were not very dramatic (Figs. 1 and 2). At other counting sites such as the contralateral and ipsilateral olfactory areas of case R2, the superiority of the TMB(m) procedure in

TABLE I

Properties of Methods

Abbreviations for Procedures	Source	Chromogen and Incubation Parameters[a]								Additional Steps	Illumination
		Chromogen	Buffer	Alcohol	Temperature	Additives	Time (min)	H_2O_2 (%)	Color		
TMB (m)	Mesulam 1978	Tetramethyl benzidine 5 mg/100 ml	Acetate 0.01M pH 3.3	2.5% Ethanol	19–23°C	Sodium nitroferricyanide	20	0.009	Blue	Pre-reaction soak 20 min at room temp stabilization 20 min at 0°C	Bright-field
DAB (sr)	Streit & Reubi 1977	Diaminobenzidine tetrahydrochloride 10 mg/100 ml	Citric ammonium acetate 0.05 M, pH 5.0	None	19–23°C	P-cresol	60	0.01	Brown	None	Dark-field
BDHC (dh)	De Olmos & Heimer 1977	Benzidine dihydrochloride 88 mg/100 ml	Acetate 0.01 M, pH 4.3	45% Methanol	−10°C	DMSO; gelatine; $CoCl_2$; sodium nitroferricyanide	330	Gradual change from 0.004 to 0.028	Blue	Pre-reaction soak 10 min at −10°C; stabilization 10 min at −10°C	Bright-field
BDHC (m)	Mesulam 1976	Benzidine dihydrochloride 50 mg/100 ml	Acetate 0.01 M, pH 5.0	30% Ethanol	19–23°C	Sodium nitroferricyanide	25	0.012	Blue	Pre-reaction soak 20 min at room temp; stabilization 20 min at 0°C	Bright-field
TMB (dhh)	De Olmos, Hardy & Heimer 1978	Tetramethyl benzidine 6.5 mg/100 ml	Acetate 0.0009 M, pH 4.3	14% Ethanol	0°C	DMSO; gelatine; sodium nitroferricyanide	60	Gradual change from 0.009 to 0.018	Blue	Pre-reaction soak in nickel ammonium sulfate 5 min at room temp	Bright-field
DAB (ll)	LaVail & LaVail 1974	Diaminobenzidine tetrahydrochloride 50 mg/100 ml	Phosphate 0.1 M, pH 7.4	None	19–23°C	None	20	0.01	Brown	Pre-reaction soak 20 min at room temp	Dark-field
O-D	De Olmos 1977	*o*-Dianisidine 38 mg/100 ml	Acetate 0.012M, pH 4.3	14% Methanol	−5°C	DMSO; ethylene glycol; sodium nitroferricyanide	120	Gradual change from 0.02 to 0.06	Green	Pre-reaction soak 10 min at −10°C; stabilization 10 min at −10°C	Bright-field
DAB (a)	Adams 1977	Diaminobenzidine tetrahydrochloride 50 mg/100 ml	Phosphate 0.1 M, pH 7.3	None	19–23°C	None	15	0.01	Blue-Brown	Pre-reaction soak in $CoCl_2$ and Tris 10 min at room temp	Dark-field
PPD-PC	Hanker, Yates, Metz & Rustioni 1977	*p*-phenylenediamine (1 part)+pyrocatechol (2 parts) 150 mg/100 ml	Tris 0.1M, pH 7.6	None	19–23°C	None	20	0.01	Brown	None	Bright-field

[a] This table summarizes the characteristics of the nine procedures employed in these experiments. The lefthand column lists the abbreviations that have been used to designate each method. The abbreviations are based on the chromogen that is being used. When more than one method uses the same chromogen, then the initial(s) of the author(s) have been added. The values listed under "Chromogen and Incubation Parameters" refer to the actual incubation solution.

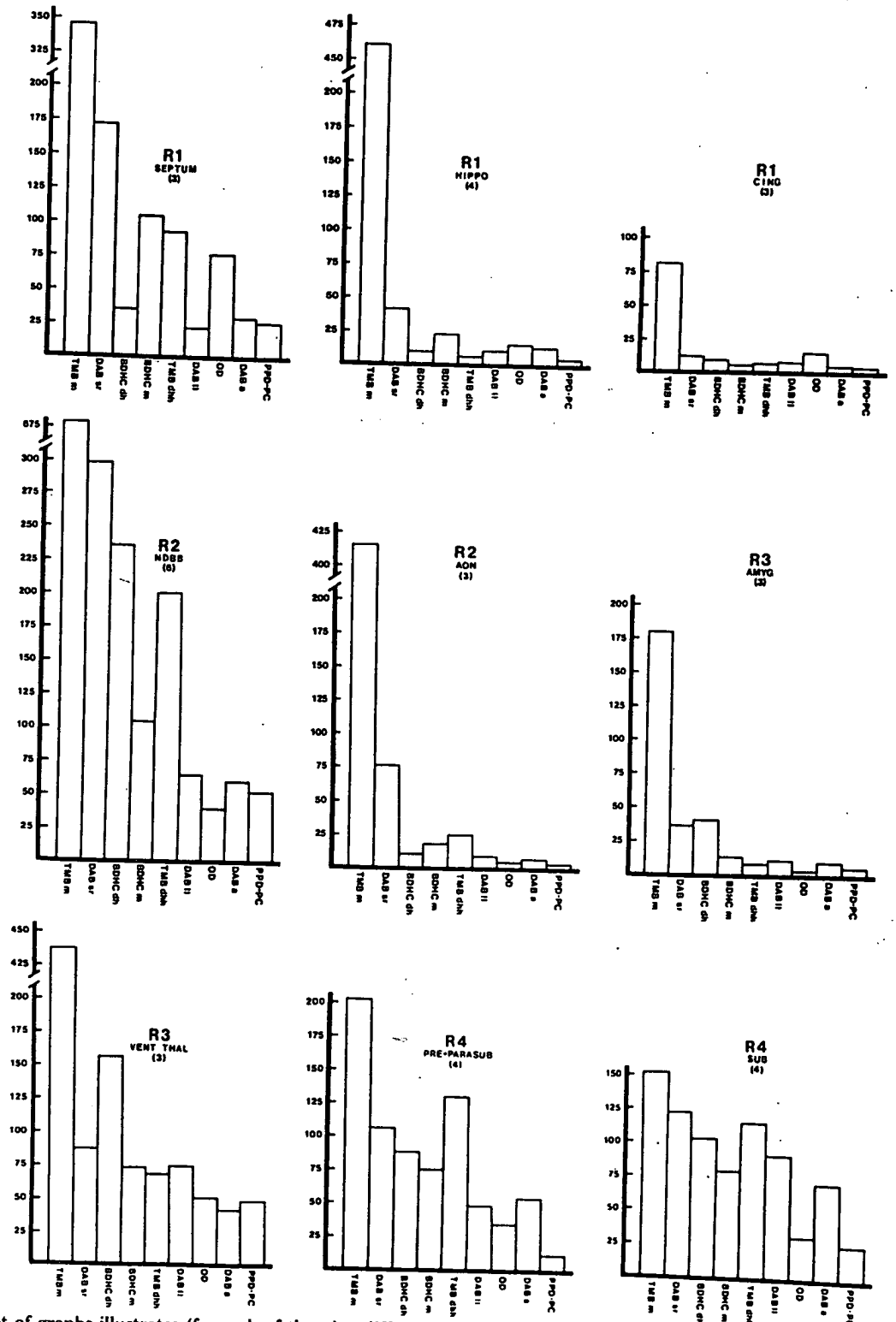

FIG. 1. This set of graphs illustrates (for each of the nine different HRP reaction methods) their relative effectiveness in demonstrating retrogradely labeled neurons in nine different afferent systems. For each graph the ordinate is the median number of HRP-labeled neurons observed at each anatomical locus. The HRP injection sites are as follows: R1-hippocampus and neocortex; R2-olfactory bulb; R3-neocortex; R4-entorhinal cortex. Below each case number on the graph is the site where HRP-labeled neurons were identified and the number of matched sections analyzed for each reaction method is given in parenthesis. These graphs illustrate the consistent superiority of the TMB(m) method as well as the relative effectiveness and variability of the other reaction methods. Note the absolute number of neurons above the interruption of the ordinate for those anatomical loci where the large number of labeled neurons found with the TMB(m) procedure could not be illustrated on a continuous scale. In the AON, HIPPO, Pre- and Parasub, the neuron count was done on the side contralateral to the injection site. Anatomical abbreviations: AMYG, amygdala; AON, anterior olfactory nuclei; CING, cingulate cortex; HIPPO, hippocampus; NDBB, nucleus of the diagonal band of Broca; Pre- and Parasub, presubiculum and parasubiculum; SUB, subiculum; VENT THAL, ventral thalamus.

TABLE II

Comparative Rank Order

Case	HRP Injection Site	Anatomical[a] Region Analyzed	N[b]	METHODS[c]								
				TMB (m)	DAB (sr)	BDHC (dh)	BDHC (m)	TMB (dhh)	DAB (ll)	O-D	DAB (a)	PPD-PC
R1	Hippocampus & neocortex	Septum (ipsi)	3	1	2	6	3	4	9	5	7	8
		Hippo (contra)	4	1	2	7	3	8	6	4	5	9
		Cing (ipsi)	3	1	3	4	7	6	5	2	8	9
R2	Olfactory bulb	NDBB (ipsi)	6	1	2	3	5	4	6	9	7	8
		AON (contra)	3	1	2	5	4	3	6	8	7	9
R3	Neocortex	Amyg (ipsi)	3	1	3	2	4	7	5	9	6	8
		Vent thal (ipsi)	3	1	3	2	5	6	4	7	9	8
R4	Entorhinal cortex	Pre- and parasub (contra)	4	1	3	4	5	2	7	8	6	9
		Sub (ipsi)	4	1	2	4	6	3	5	8	7	9
		Average rank		1.0	2.3	4.1	4.7	4.8	5.8	6.8	6.9	8.6
		Summary rank[d]		1	2	3	4	5	6	7	8	9

[a] For the anatomical regions sampled the anatomical abbreviations are the same as in Figure 1.

[b] Number of sections used for counting labeled perikarya.

[c] For each anatomical region the nine methods are ranked from 1 to 9 according to the median number of HRP labeled neurons observed. The abbreviations for the methods are identical to those in Table 1.

[d] The summary rank was obtained by ordering the methods on the basis of their average rank over all nine anatomical loci.

TABLE III

Pair-wise Independent Comparisons

Case	HRP Injection Site	Anatomical Region[a] Sampled	Number of Sections Counted	Methods[b]		P^c
M-1	Rostrum of corpus callosum	Sulcus principalis	5	TMB (m)	DAB (sr)	0.03
				26	6	
				33	3	
				14	1	
				12	2	
				11	0	
M-2	Anterior cingulate cortex	NDBB	5	TMB (m)	TMB (dhh)	0.03
				25	0	
				56	3	
				31	2	
				22	1	
				3	2	
M-3	Medial parietal cortex	Parahippocampal gyrus	5	TMB (m)	BDHC (dh)	0.03
				61	1	
				125	20	
				200	20	
				150	10	
				100	11	
M-4	Hippocampus	Septum	5	TMB (m)	BDHC (m)	0.03
				45	11	
				56	3	
				59	0	
				63	9	
				39	12	

[a] Anatomical abbreviation: NDBB - nucleus of the diagonal band of Broca.

[b] Total number of HRP-labeled neurons found in matching sections for each method. The abbreviations for the methods are identical to the ones in Table 1.

[c] Wilcoxon matched pairs, signed-rank test.

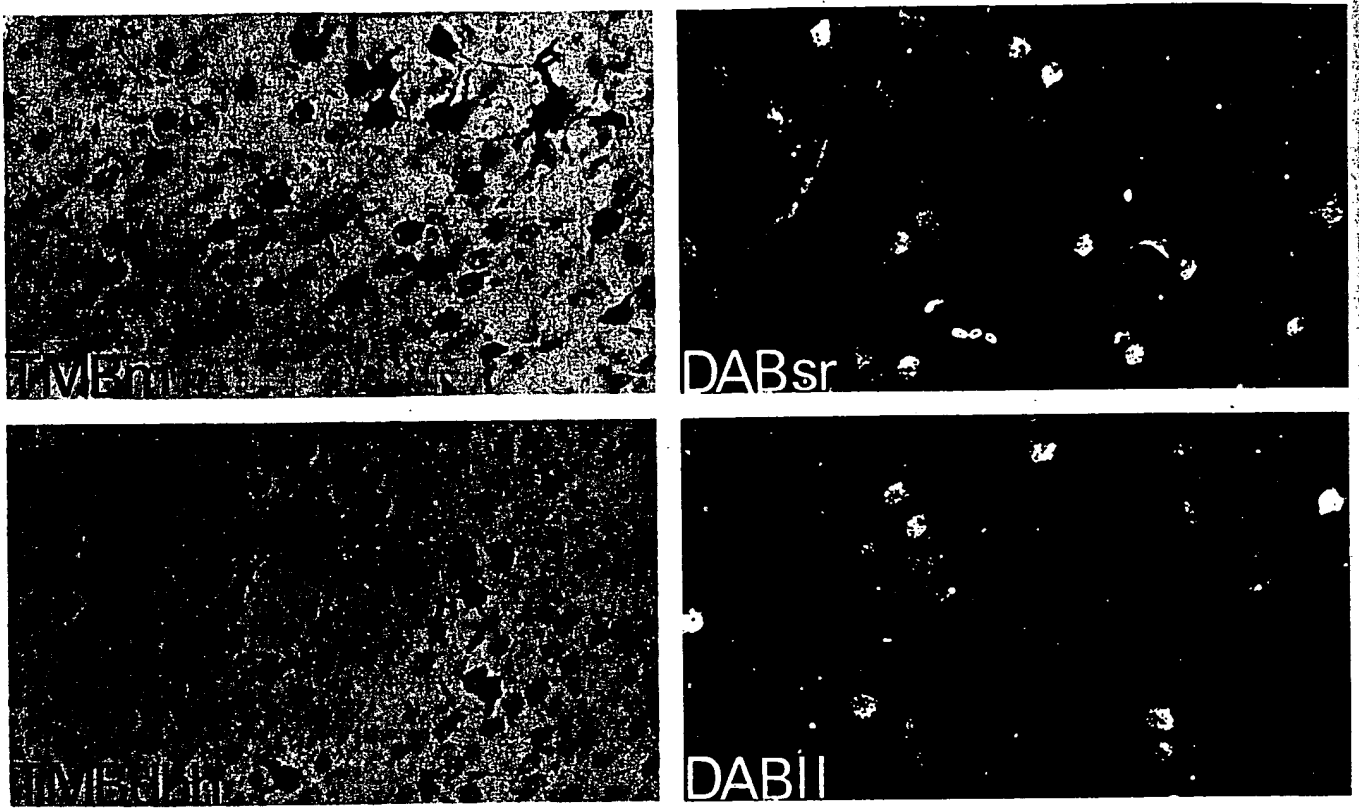

FIG. 2. These four photomicrographs are from matching sections of the subiculum in case R4. The HRP injection in this case was in the entorhinal area. Four different methods are represented and these are identified in the lower left corner. The abbreviations used here are identical to the ones in Table 1. Each of the four methods demonstrates a comparable number of perikarya. This experiment provides an example of a cerebral area where the difference among methods is not as dramatic as in many areas that were employed in the comparisons. One reason for this may be that the subicular perikarya in this case were heavily filled with HRP so that the detection of HRP in these perikarya remained above the threshold of the less sensitive methods. The photomicrographs on the left were taken under bright-field illumination and those on the right under dark-field illumination. Magnification at the film plane was ×80.

demonstrating labeled perikarya was dramatic (Figs. 1 and 3). Since for each of the 9 procedures, cases R1–R4 had been reacted simultaneously in the same reaction vessel, this variation in the magnitude of the differences cannot be attributed to surreptitious between-subject procedural differences. In fact, one variable which may explain the variation in the magnitude of the differences is the quantity of perikaryal HRP in the neurons at each of the counting sites. For instance, in the subiculum of R4, many of the perikarya were heavily filled with the HRP reaction-product. Consequently, most of the procedures demonstrated comparable numbers of labeled perikarya (Figs. 1 and 2). In contrast, the labeling in the ipsilateral primary olfactory cortex and in the contralateral anterior olfactory nuclei of R2 was light and the differences among procedures at these sites were dramatic (Figs. 1 and 3). At certain sites such as in the cingulate cortex or in the septum of Case R1, two distinct populations of neurons were labeled; one consisting of large and heavily labeled neurons and the other consisting of smaller and sparsely labeled neurons. In these instances, all 9 procedures could visualize the large and heavily labeled neurons (i.e., layer III pyramids in the cingulate cortex of R1), while only the TMB(m) procedure visualized significant numbers of the sparsely labeled neurons (i.e., those in layer VI of the cingulate cortex in R1).

In 3 of the 12 sites chosen for counting, the TMB(m) procedure was the only one which enabled the visualization of labeled neurons in significant numbers (Table 4). The neurons in these three areas (anterior hippocampal rudiment in R2, the nucleus of the lateral olfactory tract in R2 and the claustrum in R3) shared the common property of being very lightly labeled even when processed with the TMB(m) procedure. It is likely, therefore, that the small amounts of transported HRP at these three sites remained below the detection threshold of all the procedures except for TMB(m). Thus, under the specific experimental conditions which prevailed in cases R1–R3, these three connections could have been overlooked if these cases had been processed with a procedure other than TMB(m).

Anterograde transport: Since anterograde transport is not readily quantifiable, only qualitative comparisons could be made. Anterograde transport of HRP was noted in all cases. In some instances such as the neocortical injection in R3, all 9 procedures displayed anterograde transport to the thalamus. This anterograde transport was most extensive in sections processed with the TMB(m), DAB(sr), BDHC(dh), BDHC(m) and TMB(dhh) procedures. In other instances, such as the olfactory bulb injection in R2, anterograde transport to layer Ia of primary olfactory cortex was seen with

TABLE IV

Comparative Rank Order

Case	HRP Injection Site	Anatomical[a] Region Sampled	Number of Sections Counted	Methods[b]								
				TMC (m)	DAB (sr)	BDHC (dh)	BDHC (m)	TMB (dhh)	DAB (ll)	O-D	DAB (a)	PPD-PC
R-1	Hippocampus & neocortex	Claust (ipsi)	4	72	1	1	0	1	2	1	2	1
R-2	Olfactory bulb	AHR (ipsi)	3	164	8	1	0	0	0	0	1	0
		NLOT (ipsi)	2	22	0	0	0	0	0	0	0	0

[a] Total number of HRP-labeled neurons observed in matching sections processed according to the various methods. The abbreviations for the methods are identical to the ones in Table 1.

[b] Anatomical abbreviations: Claust - claustrum; AHR - anterior hippocampal rudiment; NLOT - nucleus of the lateral olfactory tract; ipsi - ipsilateral.

pr cedures TMB(m), DAB(sr), BDHC(dh), BDHC(m), TMB(dhh) and O-D, but not with procedures DAB(ll), DAB(a) or PPD-PC (Figure 3). Finally, in R1 all 9 procedures sh wed anterograde transport from the hippocampus to the septum while only the TMB(m) procedure showed the anterograde transport to the nucleus accumbens. Thus, while all 9 procedures can demonstrate some anterograde transport, the effectiveness for this parallels the sensitivity for demonstrating retr grade transport at perikaryal sites.

Injection size: The definition of an injection site is a difficult problem in HRP neurohistochemical experiments. If an injection site is merely defined as an area of dense and diffuse reaction-product deposition that is evenly distributed in the entire neuropil, these experiments showed that the more sensitive procedures demonstrated larger injection sites. In certain instances, the differences in the injection site from one procedure to another created difficulties in interpreting neural connections. For instance, in R3 only the TMB(m) and the DAB(sr) procedures demonstrated retrograde labeling in the substantia nigra. However, while the injection site in the TMB(m) procedure included neocortex as well as a dorsal rim of the caudoputamen, matching sections processed with the DAB(sr) procedure demonstrated an injection site limited to neocortex. Hence, if case R3 had only been processed with the DAB(sr) procedure, the conclusion could be reached that there is a projection from the substantia nigra to this portion of ne cortex. However, when the tissue is processed with the TMB(m) procedure, this conclusion becomes suspect since the labeled neurons in the substantia nigra could send axons exclusiv ly to the caudoputamen rather than to neocortex. The most effective means for resolving this dilemma is to study the efferent projections of the substantia nigra following injection of radiolabeled amino acids. This example illustrates the potential hazard of using HRP experiments as the sole means f r studying neural connections.

DISCUSSION

These experiments show that the effectiveness for demonstrating transported HRP depends on the nature of the histochemical procedure that is employed. In particular, the TMB(m) procedure (Table 1) is significantly superior to 8 other procedures that are currently available for demonstrating the retrograde transport of HRP in neuroanatomical investigations. These differences in sensitivity have several implications for neuroanatomical experiments. First, afferent connections of the injection site which are easily demonstrated by the TMB(m) procedure may completely elude detection by any of the other 8 procedures. This is most frequently the case if the labeled perikarya contain small quantities of transported HRP. Second, if more than one population of neurons contains perikaryal HRP, procedures which are less sensitive than TMB(m) may demonstrate reaction-product only within the population of large and heavily labeled perikarya, while a population of smaller or more sparsely labeled perikarya in the same cerebral structure may be overlooked entirely. Third, at each area containing labeled neurons, the TMB(m) procedure demonstrates a greater number of labeled perikarya and therefore indicates the presence of a more massive connection than would have been inferred on the basis of the other 8 procedures (Fig. 1).

It has been shown that the ability for demonstrating the *anterograde* transport of HRP is also dependent on the sensitivity of the histochemical procedure that is employed (Colman *et al.*, 1976; De Olmos and Heimer, 1977; Mesulam, 1976, 1978; Streit and Reubi, 1977). The comparative observations reported above clearly show that those procedures which were most successful in demonstrating retrograde transport were also most successful in demonstrating anterograde transport (Fig. 3). In particular, many sites of anterograde transport which were easily demonstrated by the TMB(m) procedure would be completely overlooked or significantly underestimated if analyzed by any of the other eight procedures. Furthermore, the traditional DAB(ll) procedure was markedly ineffective in revealing areas of anterograde transport and this may account for the earlier claims, made by investigators who employed the DAB(ll) procedure, that HRP is not significantly transported in the anterograde direction.

In addition to the demonstration of transported HRP, the size of the injection site also varied from one histochemical procedure to the other. Although exact quantitation was not attempted, a qualitative analysis indicated that those procedures which were most sensitive in demonstrating labeled perikarya also demonstrated larger injection sites. The definition of injection site is a difficult and unresolved problem in HRP neurohistochemical experiments. Ideally, it is desirable to define an "effective injection site" that precisely delimits the volume of tissue within which uptake and subsequent transport of HRP takes place. Practically, however, it is

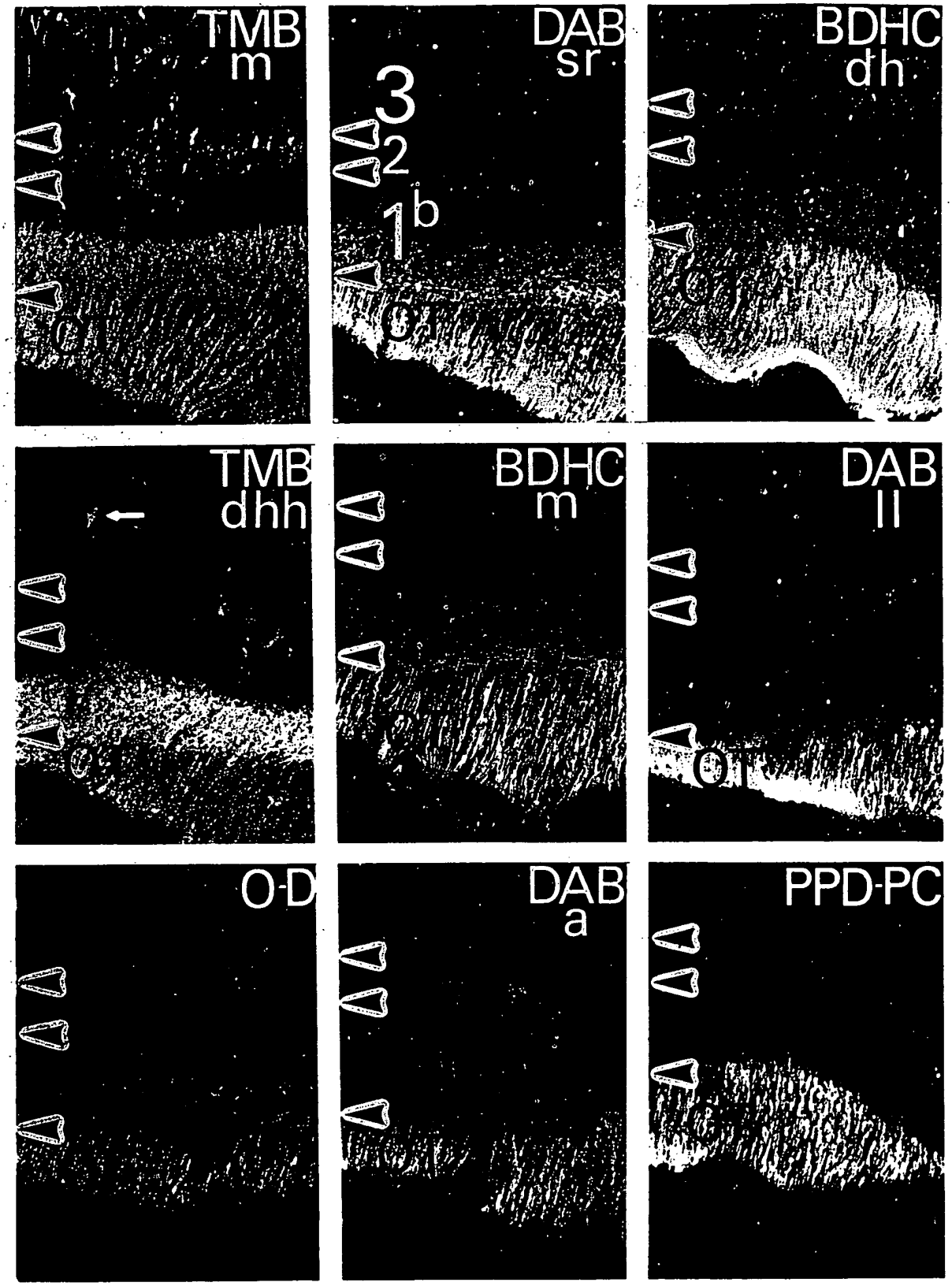

FIG. 3. Dark-field photomicrographs of matching tissue sections processed with each of the nine histochemical methods are shown in this figure. The photomicrographs depict the olfactory tract (OT) and primary olfactory cortex (POC) after an HRP injection (0.075 μl of 20% solution) in the ipsilateral olfactory bulb of case R1. The layers of POC are labeled in the middle frame of the upper row. In each of the other frames, black arrows designate the boundaries of the corresponding layers in POC. Hence, in all the photomicrographs, the two arrows just

merely possible to designate a "virtual injection site" which is identified by the presence of dense and uniform deposition of reaction-product throughout the neuropil. The "virtual injection site" has been shown to depend on survival time (Nauta *et al.*, 1974; Vanegas *et al.*, 1978), on the method of fixation (Rosene and Mesulam, 1978) and on the sensitivity of the histochemical procedure (Hardy and Heimer, 1977; Mesulam, 1978). These same parameters also affect the demonstration of transported HRP. However, it is not yet known whether or not the effect of these variables on the size of the "virtual injection site" is congruent with their effect on the extent of HRP transport that is being demonstrated. Furthermore, the rules for deducing the "effective injection site" from the observed "virtual injection site" have not yet been established. Many experimental variables may influence the magnitude and the direction of this relationship. It is conceivable that the "virtual injection site" of the more sensitive procedures overestimates the "effective injection site" by demonstrating dense deposition of reaction-product even at regions around the injection point where the actual HRP concentration had been too low to sustain sufficient uptake and transport (De Olmos *et al.*, 1978). Naturally, the converse may be true in experiments that use less sensitive methods. In view of these complicated relationships and the desirability of mapping the precise connections of the "injection site" it is conceivable that histochemical procedures employed for delineating the injection site should not have the same sensitivity as those which are employed for the detection of transport. These and alternative possibilities should be investigated in independent experiments.

These results on the comparative sensitivities of nine different methods are based on histochemical procedures as they have been executed in this laboratory. Even though the recommended methodology has been followed, it is conceivable that different results may be obtained in other hands. Moreover, all of the animals were fixed according to the procedure developed in this laboratory (Rosene and Mesulam, 1978) and it is conceivable, but unlikely, that different fixation procedures may effect individual histochemical methods differentially. Furthermore, the superiority of the TMB(m) procedure is relative to the 8 other procedures that have been used (Table 1). Although these methods adequately represent the procedures which are currently available for neuroanatomical experiments, it is conceivable that other histochemical, autoradiographic or immunological HRP procedures with even more advantageous properties may be described in the future. As far as the 9 methods performed in these experiments are concerned, the differences among their sensitivities have been established on the basis of a rigorous quantitative analysis based on the use of matching sections from the same subject in eight experimental animals, on counts of labeled perikarya by tw independent observers, and on subsequent statistical analysis f the results. Since new HRP procedures are appearing quite frequently, it would be desirable if similar methods of comparison were to be employed when substantiating claims of superior sensitivity. Furthermore, it is clear that a mere superiority over the traditional DAB(ll) procedure is no longer sufficient justification for introducing a new method since far more sensitive methods are now available.

Sensitivity is not the only consideration of importance in HRP neurohistochemical experiments: freedom from artifact, specificity of reaction-product deposition, stability of the reaction-product and suitability for electron microscopic examination are all relevant considerations. The issues of artifact, specificity and stability in the TMB(m) procedure have been discussed in detail elsewhere (Mesulam, 1978). There is virtually no experience concerning the suitability of the blue-green reaction-products for the electron microscope. Graham and Karnovsky (1966) stated that benzidine could be used for electron microscopic examination, but preferred DAB because of superior electron density. However, we are not aware of published accounts concerning the electron density of reaction-products formed with benzidine dihydrochloride (BDHC) or tetramethyl benzidine (TMB) as the chromogens. If efforts in that direction are fruitful, then the sensitivity of HRP neurohistochemistry at the electron microscopic level may be enhanced substantially. Such gains in sensitivity may increase the ability to demonstrate transported HRP within axons, boutons and dendrites and may improve the cytological analysis of neural connections. Naturally, such adaptations of the TMB(m) procedure for electron microscopic analysis may necessitate modifications that reduce the potential spread of reaction-product beyond the HRP-containing organelle. However, if it is demonstrated that DAB is essential for electron microscopic analysis, then the DAB(sr) procedure would be the method of choice since its sensitivity at the level of light microscopy significantly exceeds that of the traditional DAB(ll) procedure (Table 2). Whether or not this superiority extends into the electron microscopic level remains to be determined.

The results of these experiments suggest that the TMB(m) procedure may have a higher yield of visible reaction-product per molecule of transported HRP than any of the other 8 procedures. This ratio of visible reaction-product at the time

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above OT designate the limits of layer 1; the two closely spaced arrows designate the boundaries of layer 2, and layer 3 remains above the top arrow. The method employed in processing each of the nine sections is indicated by the abbreviation in the upper right corner. The key to these abbreviations may be found in Table 1. This figure shows the dramatic difference that exists among the different procedures. Each of the nine procedures demonstrates retrogradely transported HRP in the OT. However, only six methods, TMB(m), DAB(sr), BDHC(dh), BDHC(m), TMB(dhh) and (O-D) are able to demonstrate anterograde transport to layer 1a of POC. Furthermore, except for an isolated neuron (white arrow) in the TMB(dhh) procedure, only the TMB(m) procedure demonstrates significant numbers of retrogradely labeled neurons in layers 2 and 3 (see the photomicrograph on the upper left corner). The presence of reaction-product in the OT of sections processed with each of the nine methods provides an internal control to show that an HRP histochemical reaction had taken place in each case. Naturally, we do not wish to imply that this particular set of perikarya in layers 2 and 3 can never be shown to be labeled by methods other than the TMB(m). With differences in the injection size, in survival time, or in other variables more HRP may be transported to these perikarya so that they can then contain reaction-product when processed by less sensitive methods as well. Magnification at the film plane was ×40.

of microscopic examination to transported HRP may be defined as "sensitivity". The determinants of sensitivity in HRP histochemistry have been investigated by Straus (1964) in renal tissue, by Weir *et al.*, (1974) in immun histochemical preparations and by Streefkerk and van der Ploeg (1973) in a polyacrylamide model system. However, until recently, the role of histochemical sensitivity in n uroanatomical experiments that employ HRP has largely been ignored. The multiple factors that influence sensitivity in an HRP neurohistochemical experiment include the method of fixation, the details of postfixation storage, the nature of the chromogen, the choice of incubation parameters, the type of exogenous HRP that is administered and the postreaction treatment (Adams, 1977; Bunt *et al.*, 1976; Courville and Saint-Cyr, 1978; De Olmos, 1977; De Olmos and Heimer, 1977; Kim and Strick, 1976; Malmgren and Olsson, 1978; Mesulam, 1976, 1978; Ogren, 1977; Rosene and Mesulam, 1978). Two of these factors, the choice of chromogen and of incubation parameters are of particular relevance to these experiments.

One effect of the chromogen is to determine the color and hence the visibility of the reaction-product. Reaction-products with better visibility tend to enhance sensitivity even though this is not without exception as in the case of the light-brown reaction-product in the DAB(sr) procedure which affords better sensitivity than the BDHC(m) procedure despite the dark-blue reaction-product of the latter (Table 2). There are other properties of the chromogen such as lipid solubility, oxidation potential, tendency to polymerize and precipitate, chemical stability and propensity to diffuse from sites of deposition, all of which may influence sensitivity. For instance, despite comparable visibility, TMB as the chromogen offers much better sensitivity than BDHC, even when incubation parameters which are optimal for each are being utilized (Mesulam, 1978). The exact determinants of this difference are not understood. It is conceivable that the superiority of TMB may depend, at least in part, on its higher lipid solubility which makes it more accessible to the organelle-bound HRP.

Sensitivity is also determined by the choice of incubation parameters (Mesulam, 1976, 1978). Even if the fixation procedure is appropriate and a superior chromogen such as TMB is employed, failure to choose optimal incubation parameters results in a lowering of sensitivity. Indeed, the differences between the TMB(m) and TMB(dhh) procedures are due to differences in incubation parameters. A parametric analysis of incubation variables in procedures using TMB as the chromogen has indicated that sensitivity was depressed by an incubation pH higher than 4, an excessive alcohol concentration in the incubation medium or a low reaction temperature (Mesulam, 1978). The incubation variables of the TMB(m) procedure were selected on the basis of these observations. A comparison of these parameters in Table 1 shows that the pH 4.3, 14% alcohol concentration and 0°C reaction temperature of the TMB(dhh) procedure are distinctly different from the pH 3.3, 2.5% alcohol concentration and 21°C reaction temperature of the TMB(m) procedure. These differences in incubation parameters would be expected to effect sensitivity and it is clear that the higher chromogen c ncentrati n and longer reaction times of the TMB(dhh) procedure have n t been able to compensate for the differences in pH, alcohol concentration and reaction temperature (Tabl 2). Optimal incubation parameters must be determined independently f r each chromogen and cannot be deduced from the *in vitro* kinetics of the HRP + $H_2O_2 \rightleftharpoons [HRP \cdot H_2O_2]$ reaction al ne since the objective is not merely to maximize the enzymatic action of HRP on H_2O_2, but also to xidize, polymerize and precipitate large quantities of a chrom gen with individual chemical properties and to keep this precipitate at tissue sites c ntaining HRP activity.

In summary, these experiments demonstrate that the extent of HRP transport which is demonstrated depends on th choice of histochemical parameters. While the superiority of the TMB(m) method for demonstrating anterograde and retrograde transport is largely due to the choice of chromogen and incubation parameters, the method of fixation, the postfixation storage, the type of injected HRP and the details of post-reaction handling also determine the overall sensitivity of any HRP neurohistochemical experiment. A thorough understanding and adequate manipulation of these variables can result in HRP neurohistochemical experiments which optimize both the amount and the quality of information obtained.

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